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Primary sequence determinants responsible for site selective dephosphorylation of the PDGF β-receptor by the receptor-like protein tyrosine phosphatase DEP-1

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Abstract Site-selective dephosphorylation of receptor tyrosine kinases contributes to receptor regulation. The receptor-like protein tyrosine phosphatase DEP-1 site-selectively dephosphorylates the PDGF β-receptor. DEP-1 dephosphorylation of original and chimeric phospho-peptides spanning the preferred pY1021 and the less preferred pY857 and pY562 sites was analyzed. Double substitutions of basic residues at −4 and +3 of pY857 and pY562 peptides improved affinity. Substitutions of single amino acids indicated preference for an acidic residue at position −1 and a preference against a basic residue at position +3. DEP-1 site-selective dephosphorylation of PDGF β-receptor is thus determined by the primary sequence surrounding phosphorylation sites and involves interactions with residues spanning at least between positions −1 and +3.

Key words: Protein tyrosine phosphatase; Substrate specificity; Phospho-peptide; DEP-1; PDGF β-receptor

1. Introduction

Signaling through receptor tyrosine kinases (RTKs) is a major mechanism for intracellular communication in physiological and pathological settings [1]. Activation and autophosphorylation of most RTKs are triggered by ligand-induced receptor dimerization [2,3]. Phosphorylated tyrosine residues of RTKs regulate the intrinsic tyrosine kinase activity or act as docking sites for signaling proteins containing Src homology 2 (SH-2) or phosphotyrosine-binding domains. For example, in the PDGF β-receptor, phosphorylation of Tyr857 regulates tyrosine kinase activity whereas phosphorylation of Tyr1021 creates a binding site for phospholipase C-γ [4]. RTK phosphorylation is also regulated by the action of protein tyrosine phosphatases (PTPs) [5]. Dephosphorylation of RTKs by PTPs can lead to general attenuation of receptor signaling, or modulation of signaling through site-selective dephosphorylation.

DEP-1 (also designated CD148 or rPTP-η) is a receptor-like PTP expressed in many cell types including fibroblasts, hematopoietic cells, endothelial cells and epithelial cells [6–8]. Structurally, DEP-1 is composed of an extracellular domain of eight fibronectin type III repeats, a transmembrane domain and an intracellular PTP domain. DEP-1 expression is upregulated by increasing cell density in endothelial cells, smooth muscle cells and fibroblasts [6,8]. DEP-1 negatively regulates T-cell receptor activation through a mechanism that involves reduced phosphorylation of phospholipase C-γ and LAT [9–11]. Loss of DEP-1 expression in thyroid cancer cells and breast cancer cells, and observations of growth reduction or differentiation of cancer cells after reconstitution of DEP-1 expression, suggest a possible function of DEP-1 as tumor suppressor [12–14]. The activity of DEP-1 can be regulated by ligand-binding; it was recently shown that Matrigel, a commercial preparation of extracellular matrix components, contains a DEP-1 agonist [15].

DEP-1 site-selectively dephosphorylates the PDGF β-receptor in vitro as well as in intact cells [16]. This preference for individual phosphorylation sites was reproduced in peptide dephosphorylation experiments with PDGF β-receptor-derived phospho-peptides. These observations point to the interesting possibility that the signaling output of the PDGF β-receptor, and ultimately the biological effects triggered by receptor activation, is controlled by site-selective PTPs including DEP-1. To provide a better understanding of this poorly understood regulatory phenomenon, we have in this study investigated the role of the primary sequence, surrounding preferred and less preferred phosphorylation sites in the PDGF β-receptor, in determining DEP-1 site-selectivity.

2. Materials and methods

2.1. Expression and purification of DEP-1 catalytic domain

The segment encoding the catalytic domain (amino acids 997–1337) of DEP-1 (DEP-1cd) was cloned into the expression vector PET-15b (Novagen). Protein expression was performed in *Escherichia coli* (BL21) grown in 1 l of LB supplemented with 50 μg/ml ampicillin. At an optical density of 0.3 at 600 nm, 1 mM isopropyl-β-D-thiogalactopyranoside was added. The bacteria were cultured for 3 h at 30°C, collected by centrifugation and resuspended in 14 ml of buffer A (5 mM imidazole, 0.5 M NaCl and 20 mM Tris pH 7.9) supplemented with two protease-inhibitor cocktail tablets Complete™ Mini EDTA-free (Roche). Lysis was performed by sonication three times for 30 s at 35% power (Bandelin sonopuls). The lysate was centrifuged (39 000×*g* for 20 min) and the supernatant applied to 4 ml nickel-charged Sepharose (Novagen). The Sepharose was washed with 100 ml of buffer A and DEP-1cd eluted with 24 ml of buffer A containing 0.1 M imidazole. The eluted fraction was dialyzed overnight against buffer B (20 mM Tris–HCl pH 8.0, 1 mM Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), 2 mM benzamidine and 2 mM EDTA),

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and applied to a Mono Q 5/5 column (Amersham Pharmacia Biotech). The column was developed with a linear gradient of 0–1 M NaCl in 20 ml of buffer B. DEP-1cd eluted between 100 and 140 mM NaCl. DEP-1cd-containing fractions were pooled and loaded onto a Superdex 75 16/60 gel filtration column (Amersham Pharmacia Biotech) equilibrated in 20 mM Tris–HCl pH 8.0 and 1 mM TCEP. The fractions containing DEP-1cd, which eluted at 63–72 ml, were pooled and concentrated to approximately 5 mg/ml. Amino acid analysis was used for determination of the concentration of purified DEP-1cd. The protein was stored at −20°C.

2.2. Peptide synthesis and in vitro phosphatase assay

All peptides were synthesized following the Fmoc strategy with Fmoc-amide resin, resulting in carboxy-terminally amidated peptides. For phosphotyrosine, Fmoc-Tyr(PO_3H_2)-OH (Novabiochem) was used. Peptides were purified by reversed phase high-performance liquid chromatography using a C18 column, and matrix-assisted laser desorption ionization–time of flight–mass spectrometry analysis confirmed the correct, expected molecular weights. Concentrations of peptides were determined by measuring differences in absorbance at 282 nM of phosphorylated and fully dephosphorylated peptides. The dephosphorylation of phosphotyrosine-containing peptides was measured spectrophotometrically by continuously monitoring the increase in absorbance at 282 nm [17]. Initial rates were determined from the graphs obtained. All measurements were performed at 30°C in 500 μl of a buffer containing 20 mM Tris–HCl pH 7.5, 0.15 M NaCl, 1 mM EDTA and 2 mM 1,4-dithiothreitol. The peptide concentration ranged from 50 to 5000 μM, which is within the linear range of absorbance. Kinetic parameters were obtained from Lineweaver–Burk plots using the program Enzyme Kinetics (Trinity Software), which uses a concentration-dependent weighting scheme. In some experiments dephosphorylation rates were measured by determining the amount of phospho-peptide (starting concentration of 50 μM) remaining after 25 s of incubation with 50 nM DEP-1cd.

3. Results

3.1. Purification of DEP-1 catalytic domain

The catalytic domain of DEP-1 (DEP-1cd) comprising amino acids 997–1337 was expressed as an amino-terminal histidine-tagged protein in *E. coli* cells. The recombinant protein was purified from the soluble fraction of the cell lysate by metal affinity chromatography followed by ion-exchange chromatography and gel filtration. Fractions from the different purification stages were analyzed by SDS–PAGE and Coomassie blue staining (Fig. 1). The purity of final DEP-1cd was estimated to be >90% as determined from SDS–PAGE; the yield from 1 l of culture was approximately 5 mg.

3.2. Difference in DEP-1cd dephosphorylation efficiency of the pY857 and pY1021 peptides is caused by a difference in affinity

It has previously been shown that DEP-1 site-selectively dephosphorylates the PDGF β-receptor [16]. One of the most preferred sites was pY1021 whereas pY857 was found to be a poorer substrate. To further characterize pY1021 and pY857 as substrates for DEP-1, phosphotyrosine decapeptides containing these sites were synthesized (Table 1). The dephosphorylation was assayed by measuring the increase in absorbance at 282 nm and kinetic parameters were determined from Lineweaver–Burk plots (Fig. 2A). Both phospho-peptides pY1021 and pY857 exhibit similar k_{cat} values of 770 s^{-1} and 1100 s^{-1}, respectively. However, the K_m for pY1021 is 10-fold lower than that for pY857 (480 μM and 4800 μM, respectively). This analysis thus reveals that the previously observed preference of DEP-1 for the pY1021 peptide, as compared to the pY857 peptide, is caused by a much higher affinity for pY1021 rather than a higher k_{cat} value.

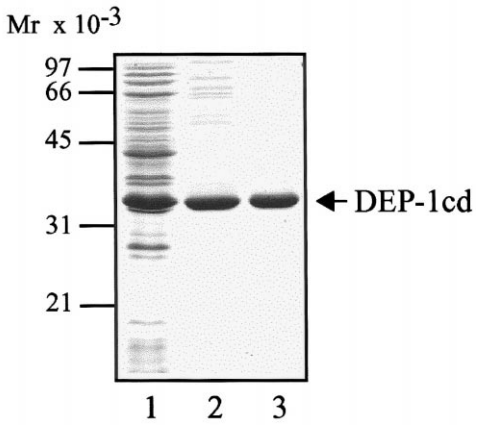

Fig. 1. SDS–PAGE analysis of purification of DEP-1 catalytic domain. The catalytic domain of DEP-1 (DEP-1cd) was expressed in *E. coli*. Cell lysate (lane 1) was applied to a nickel-charged Sepharose column and the eluted fraction (lane 2) was further purified by ion-exchange chromatography, followed by size exclusion chromatography (lane 3). Fractions were analyzed by SDS–PAGE and Coomassie blue staining.

3.3. DEP-1cd substrate selectivity is reversed for chimeric pY857/1021 peptides containing substitutions in positions −4 and +3

Besides pY857, the phosphorylation site pY562 in the PDGF β-receptor has been shown to be a poor substrate for DEP-1 [16]. Common to these two sites are basic amino acid residues in positions −4 and +3 relative to the phosphotyrosine (Table 1). In order to investigate if these amino acid residues are responsible for the negative selection by DEP-1, two peptide chimeras were synthesized. In one peptide (pY857GP), the amino acid residues in positions −4 and +3 were replaced with the corresponding residues from the pY1021 peptide. Conversely, the equivalent positions in the pY1021 peptide were substituted with residues from the pY857 peptide (pY1021RK; Table 1). The k_{cat} values of the chimeric peptides remained unchanged compared to the original peptides. However the K_m of pY857GP was decreased about four-fold while a four-fold increase in K_m for the pY1021RK peptide was observed (Fig. 2B). These findings suggest that the arginine and/or lysine residues in positions −4 and +3 contribute to the poor dephosphorylation of pY857 by DEP-1cd.

3.4. The pY562 peptide is improved as a DEP-1cd substrate by substitutions with amino acids from pY1021 peptide in positions −4 and +3

To investigate if the improvement of pY857 as a DEP-1cd substrate could be reproduced for the pY562 phosphorylation site, a pY562 phospho-peptide with substitutions from pY1021 peptide in positions −4 and +3 was synthesized (Table 1). Since the corresponding pY562 decapeptide contains an amino-terminal glutamine residue, an additional amino acid residue was added to avoid the formation of a pyroglutamate. The high background absorbance of pY562 and pY562GP, due to the presence of two tryptophan residues, prevented analysis over a broad range of substrate concentrations and subsequent calculations of kinetic parameters. Instead, dephosphorylation was performed at a fixed substrate concentration of 50 μM, and the amount of phospho-peptide left after 25 s was determined. The fraction of phospho-pep-

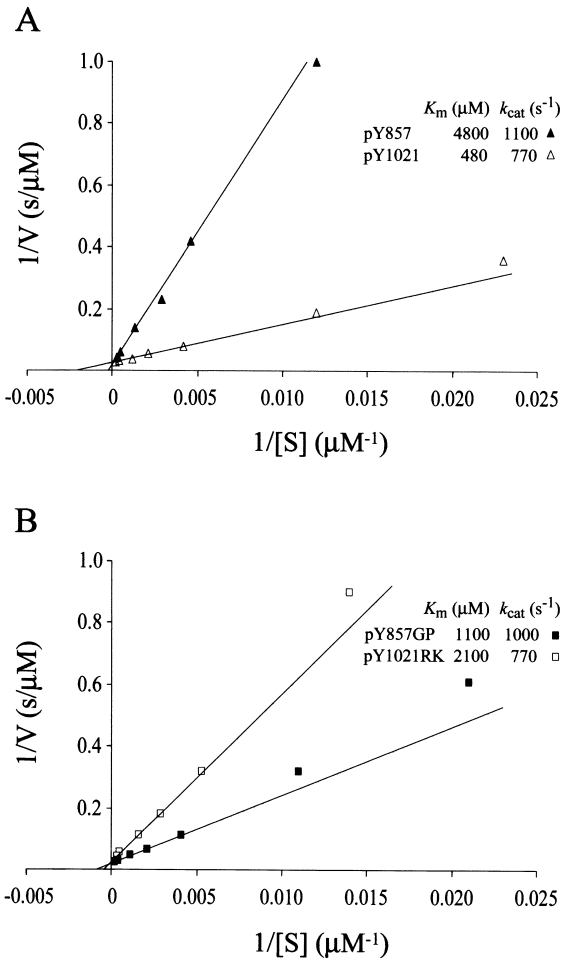

Fig. 2. DEP-1cd-catalyzed dephosphorylation of phospho-peptides pY1021, pY857, pY857GP and pY1021RK. DEP-1cd at a concentration of 50 nM was incubated with 50–4550 μM of the phospho-peptides and dephosphorylation measured spectrophotometrically. Lineweaver–Burk plots and kinetic parameters for DEP-1cd dephosphorylation of peptides pY857 and pY1021 (A) and peptides pY857GP and pY1021RK (B) are shown. The results are representative for five experiments.

tide pY562 left after incubation of DEP-1cd was 71% whereas the fraction remaining of phospho-peptide pY562GP was only 47% (Fig. 3). Similarly, pY857, which was included in this assay as a reference peptide, was dephosphorylated less efficiently than pY857GP, in agreement with the results in Fig. 2. This indicates that the presence of basic amino acid residues in positions −4 and +3 also contributes to the low dephosphorylation of the pY562 peptide by DEP-1.

3.5. The introduction of alanine residues in positions −4 and +3 in pY857 peptide improves the peptide as a DEP-1cd substrate

In order to determine if the observed improvement in dephosphorylation efficiency of pY857GP and pY562GP is due to the removal of the positively charged arginine and lysine residues, or to the specific introduction of glycine and proline residues, a pY857 peptide containing alanine residues in positions −4 and +3 (pY857AA; Table 1) was synthesized and analyzed as DEP-1cd substrate. While all peptides exhibited similar k_{cat} values, the K_m of pY857AA was decreased about three-fold compared to pY857. As shown in Fig. 4, the K_m of pY857AA was almost as low as that of pY857GP (1300 μM and 1200 μM, respectively). Hence, the increase in affinity of pY857GP, as compared to the original pY857 peptide, for DEP-1cd is most likely due to the removal of positively charged amino acid residues rather than the specific introduction of residues from the pY1021 peptide in positions −4 and +3.

3.6. The lysine residue in position +3 contributes most to the low affinity of pY857

To further examine to what extent each individual amino acid surrounding the phosphotyrosine in pY1021 contributes to DEP-1cd selectivity, chimeric peptides were synthesized in which single amino acid residues in pY857 were substituted with residues in pY1021 at position −4 throughout position +3 (pY857G, pY857N, pY857D, pY857I, pY857P; Table 1). Kinetic parameters of the DEP-1cd-catalyzed dephosphorylation of the peptides were determined (Table 2) except for pY857I, which exhibited poor solubility. A slight decrease in K_m of pY857G and pY857N could be observed whereas the K_m of pY857D was decreased two-fold. The greatest increase in affinity was shown for pY857P with a K_m (1600 μM) nearly as low as for pY857GP (1200 μM). No dramatic changes of the k_{cat} values were observed.

Furthermore, single amino acid residues at positions −4 and +3 in pY1021 were substituted with residues in pY857 (pY1021R and pY1021K; Table 1). Only pY1021R was subjected to phosphatase assays since pY1021K displayed low solubility. The K_m of pY1021R was only slightly higher than for the original pY1021 peptide, indicating that the four-fold lowered affinity of pY1021RK is mainly caused by the introduction of the lysine in position +3.

From these findings, we conclude that the lysine residue in position +3 in pY857 is a major negative determinant for the dephosphorylation by DEP-1cd.

3.7. Introduction of a lysine residue in position +2 of pY1021 reduces affinity for DEP-1cd

Basic amino acid residues at all positions between −4 and +4 relative to the phospho-tyrosine have previously been shown to be detrimental for peptide dephosphorylation by

Table 1
Amino acid sequence of PDGF β-receptor-derived tyrosine-phosphorylated peptides

Peptide	Sequence
pY857	MRDSNpYISKG
pY1021	EGDNDpYIIPL
pY562	WQKKPRpYEIRW
pY857GP	M**G**DSNpYIS**P**G
pY857AA	M**A**DSNpYIS**A**G
pY857G	M**G**DSNpYISKG
pY857N	MRD**N**NpYISKG
pY857D	MRDS**D**pYISKG
pY857I	MRDSNpYI**I**KG
pY857P	MRDSNpYIS**P**G
pY1021RK	E**R**DNDpYII**K**L
pY1021R	E**R**DNDpYIIPL
pY1021K	EGDNDpYII**K**L
pY1021K+2	EGDNDpYI**K**PL
pY1021K+1	EGDNDpY**K**IPL
pY562GP	WQ**G**KPRpYEI**P**W

Bold letters indicate substituted amino acids.

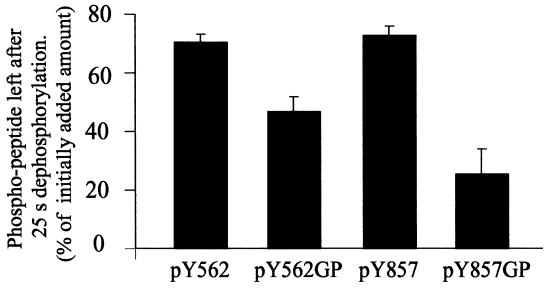

Fig. 3. Dephosphorylation of phospho-peptides pY562, pY562GP, pY857 and pY857GP by DEP-1cd. 50 nM of DEP-1cd was incubated with the phospho-peptides at a starting concentration of 50 μM and the fraction of phospho-peptide left after 25 s was determined. Data are means of three separate experiments. Error bars indicate standard deviation.

PTP1B [18]. To investigate if basic residues at other carboxy-terminal positions than +3 also act as negative determinants for DEP-1cd, lysines were introduced at positions +1 and +2 in pY1021 (pY1021K+1 and pY1021K+2; Table 1). The introduction of a lysine in position +1 had only a minor effect on affinity, increasing K_m less than two-fold. Lysine in position +2 resulted in a three-fold increase in K_m. Hence, in addition to position +3, lysine in position +2 is poorly tolerated by DEP-1cd whereas a basic residue in position +1 is better accepted.

4. Discussion

In summary, a lysine at position +3 was identified as a negative determinant of DEP-1 preference and an aspartic acid residue at position −1 was found to be a positive determinant. More generally, our study shows that the preference displayed by DEP-1 is dictated by sequences extending at least as far as position +3.

Basic residues in the vicinity of the phospho-tyrosine have also been found to act as negative determinants in the specificity of other PTPs, e.g. PTP-1B and T-cell phosphatase

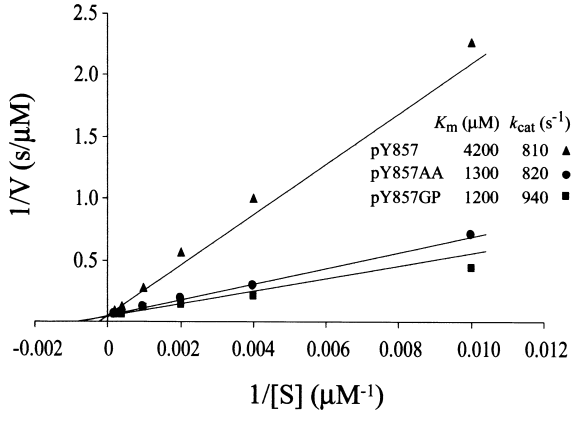

Fig. 4. DEP-1cd-catalyzed dephosphorylation of phospho-peptides pY857, pY857AA and pY857GP. DEP-1cd at a concentration of 25 nM was incubated with the phospho-peptides pY857, pY857AA and pY857GP at 100–5000 μM and dephosphorylation measured spectrophotometrically. Results are shown as Lineweaver–Burk plots and kinetic parameters indicated. The results are representative for two experiments.

[18,19]. The negative effect of a basic residue carboxy-terminal of the phospho-tyrosine appears to be position-dependent since smaller effects were observed when a lysine was introduced at position +2, and the effects were still smaller when the lysine was at the +1 position. This is thus in contrast to observations for PTP-1B specificity where selection against basic residues was observed at all positions between +1 and +4 [18]. The preference for negatively charged residues at position −1 has also been described for PTP-1B [20].

Structural studies of peptide/PTP complexes have provided the most detailed information concerning molecular interactions determining PTP specificity. Such complexes are available for PTP-1B and the peptides DADEpYL, ELEFpYMDE, and ETDpYpYRKGGKGLL, as well as for SHP-1 and the SIRPα peptides EDTLTpYADLD and PSFSEpYASVQ [21–24]. The regions of the PTPs involved in peptide interactions cluster around the α1/β1-loop and the α5-loop-α6 region, and in the case of the SHP-1/EDTLTpYADLD complex, the β5-loop-β6, as well (reviewed in [25]). The PTP-1B complexes display specific side chain/side chain interactions with residues at positions −4 to −1 and +1. Substrate residues in the +2 and +3 positions have been placed at poorly defined locations distant from the protein. Our study points to the importance of residues in position +3, which suggests that specific interactions occur at positions not yet identified in these peptide/PTP complexes. This idea is supported by a study of PTP-1B specificity, where significant differences in k_{cat}/K_m values were observed between peptides varying exclusively in position +3 [18].

The complexes between SHP-1 and the two SIRPα peptides are reported to have a very different docking of the peptide as compared to the PTP1B/peptide complexes; a +3-binding pocket was identified, composed of Ser498, Ile281 and Lys259 [24]. At the corresponding positions in DEP-1 are proline, valine and serine residues, respectively, residues which would not be expected to specifically disfavor binding of a positively charged side chain. The available structural data, therefore, do not indicate which regions in DEP-1 are responsible for the preference against basic residues at position +3.

The issue of preference for various PDGF β-receptor-derived phospho-peptides has been investigated with the IF2 isoform of LMW-PTP, another enzyme with PTP activity that has been implicated in control of PDGF β-receptor signaling [26]. This enzyme, which is a member of the structurally distinct low-molecular weight PTP family, displayed a reverse preference compared with DEP-1, i.e. the affinity of

Table 2
Kinetic parameters of pY857, pY1021 and chimeric pY857/1021 peptides

Peptide	K_m (μM)	k_{cat} (s^{-1})
pY857	4200	810
pY1021	480	710
pY857GP	1200	940
pY1021RK	2300	560
pY857G	3400	830
pY857N	3600	820
pY857D	2200	800
pY857P	1600	870
pY1021R	540	720
pY1021K+1	760	740
pY1021K+2	1400	840

The values are representative for two experiments.

the IF2 isoform of LMW-PTP was higher for the pY857 phospho-peptide than for the pY1021 phospho-peptide. Thus, the preference among PDGF β-receptor-derived phospho-peptides that DEP-1 displays is not shared by all enzymes involved in PDGF β-receptor dephosphorylation.

Our findings are consistent with a role for regulated site-selective dephosphorylation in fine-tuning of the signaling of PDGF β-receptor, and possibly other signaling proteins containing multiple tyrosine phosphorylation sites. The biological response to PDGF receptor activation is determined by the strength and duration of signaling emanating from SH-2 domain-containing enzymes recruited to the receptor. It is thus an interesting possibility that the regulated action of different site-selective PTPs represents a mechanism for altering the biological responses following ligand stimulation of PDGF receptors or other tyrosine kinase receptors. The availability of cell lines derived from mice with genetically inactivated PTPs acting on the PDGF receptor should make it possible to test this hypothesis.

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